AFFINITY LABELING OF PROTEINS AT THE mRNA BINDING SITE OF RAT LIVER RIBOSOMES BY AN ANALOGUE OF OCTAURIDYLATE CONTAINING AN ALKYLATING GROUP ATTACHED TO THE 3'-END

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1. Introduction

Affinity labeling of *Escherichia coli* ribosomes by means of a number of different analogues of mRNA has demonstrated that ribosomal proteins S1, S4, S10, S18 and S21 are located at or very close to the mRNA binding site of the small ribosomal subunit [1–4]. A heptauridylate containing the same alkylating group as that used in the present study labeled specifically ribosomal proteins S18, S9 and S1 [5].

Information on eukaryotic ribosomal proteins participating in mRNA binding is still scarce. There is some evidence from UV irradiation experiments that protein S6 is involved in poly(U) binding [6].

This study reports on affinity labeling experiments carried out with 80 S and 40 S ribosomal particles from rat liver and the 4-(*N*-2-chloroethyl-*N*-methylamino)-benzaldehyde [^{14}C]acetal derivative of octauridylate [$(pU)_8$–RCl] containing the alkylating group in the 3'-position. Binding of $(pU)_8$–RCl to ribosomal particles was increased by addition of uncharged tRNA. After completion of the reaction at 25°C for 20 h the bulk of radioactivity was found associated with ribosomal protein(s) S3/3a* which therefore seem(s) to be located, at least in part, very close to the entrance site of mRNA into the ribosomal A-site.

2. Materials and methods

Ribosomes were freed of mRNA and nascent protein [8] and 40 S ribosomal subunits were prepared by zonal centrifugation [9]. Immediately before use β-mercaptoethanol was removed from the particles by gel filtration on Sephadex G-25 equilibrated with 10 mM triethanolamine (TEA), 50 mM KCl, 5 mM $MgCl_2$ (pH 7.5).

The 4-(*N*-2-chloroethyl-*N*-methylamino)-benzaldehyde [^{14}C]acetal derivative of octauridylate [$(pU)_8$–RCl] was obtained from Dr Karpova [5,10]; it had spec. act. 0.45 GBq/mmol.

E. coli tRNA was purchased from Boehringer (Mannheim) and if necessary charged with [^{3}H]phenylalanine (703 GBq/mmol; Radiochemical Centre, Amersham) [8]. For binding experiments, as shown in fig.1, the α-amino group of Phe-tRNA was acylated by 2-nitro-4-azidobenzoyl-*N*-hydroxysuccinimide ester resulting in NAB-Phe-tRNA [8].

In the affinity labeling experiments ribosomes or small ribosomal subunits at 5–10 mg/ml were incubated for 20 min at 25°C with a 15–20-fold molar excess of $(pU)_8$–RCl in buffer A (10 mM TEA, 100 mM KCl and 18 mM $MgCl_2$ (pH 7.5)) containing uncharged tRNA (0.8 mg/1mg 80 S ribosomes or 1.7 mg/1mg 40 S subunits, respectively). After incubation samples were passed through columns of Sephadex G-50 equilibrated with buffer A at 4°C and the fractions containing the (pU)–RCl charged ribosomal particles were incubated for 20 h at 25°C in the presence of 5 μl toluene/ml. β-Mercaptoethanol to 70 mM, and 0.8 vol. ethanol containing 50 mM $MgCl_2$ were then added, and after 30 min at −20°C precipitates of the ribosomal particles were pelleted by centrifugation for 20 min at 15 000 × *g*. Pelleted 80 S ribosomes were dissociated by resuspension in 10 mM TEA, 500 mM KCl, 1mM $MgCl_2$, 10 mM β-mercaptoethanol (pH 7.5) to ~10 mg ribosomes/ml, and incubation for 5 min at 37°C and the subunits were then sepa-

* According to the new proposed international nomenclature [7] used throughout

rated on 10–30% sucrose gradients in buffer B (10 mM TEA, 500 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol (pH 7.5)) in a Spinco SW 25.2 rotor at 20°C. The fractions containing 40 S and 60 S subunits, respectively, were pooled and the particles isolated by ethanol precipitation (see above). For isolation of ribosomal proteins the individual particle fractions obtained were dissolved in a small volume of water (5–10 mg/ml) and 2 vol acetic acid containing 50 mM $MgCl_2$ (pH 1) were added. The samples were then incubated for 60 min at 30°C and thereafter for 30 min at 0°C in order to achieve complete hydrolysis of the acetal bond between the ribose moiety of $(pU)_8$ and the reacted group carrying the radioactivity. As a consequence the radioactivity, but not the uridylate residues, remains with the reacted ribosomal components and hence the electrophoretic mobility of the substituted ribosomal proteins is not influenced significantly. Further preparation and analysis of ribosomal proteins followed the usual scheme [11,12].

Poly(U) >100 000 M_r was prepared according to [13]. In competition experiments 5–7 mg/ml poly(U) were given in addition to the usual amount of $(pU)_8$–RCl (see above).

3. Results

3.1. *Template activity of octauridylate*

As the first prerequisite for the affinity labeling experiments the template activity of $(pU)_8$ was compared with that of polyuridylic acid with respect to the non-enzymic binding of N-substituted [^{3}H]Phe-tRNA (NAB-Phe-tRNA) at 18 mM $MgCl_2$. As is obvious from fig.1, maximal $(pU)_8$-directed binding of NAB-Phe-tRNA to ribosomes is ~17% of that observed with poly(U). The lower template activity of the short-length oligo- or polynucleotides in comparison to the longer polynucleotides is in agreement with earlier findings on polypeptide synthesis [14].

3.2. *Addition of uncharged tRNA*

The relatively low binding efficiency of $(pU)_8$–RCl to ribosomal particles could be considerably enhanced by the addition of uncharged tRNA (table 1) to the incubation mixture. A very similar effect was previously observed in a bacterial system [5]. As a consequence of tRNA addition most of the $(pU)_8$–RCl bound can be expected to be positioned with 3 uridylate residues in the ribosomal P-site.

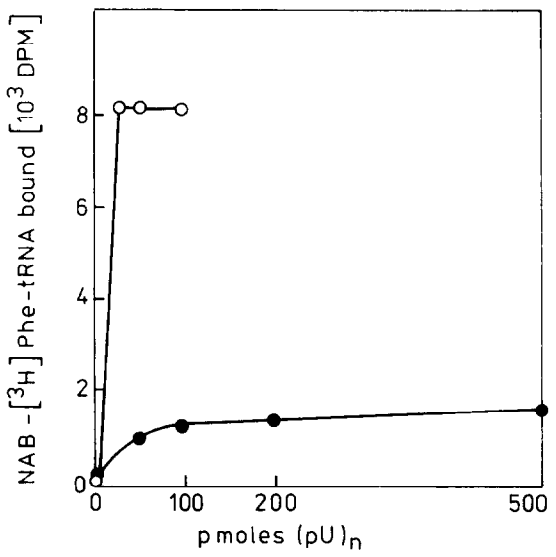

Fig.1. Binding of NAB-[^{3}H]Phe-tRNA to 80 S ribosomes as a function of poly(U) and octauridylate. Ribosomes, 50 μg (11 pmol) were incubated for 15 min at 37°C with the indicated amounts of $(pU)_{>300}$ (o) or $(pU)_8$ (•) in the presence of 10 mM TEA, 100 mM KCl, 18 mM $MgCl_2$ (pH 7.5) in 100 μl. Samples were filtered through nitrocellulose filters (Fa. Sartorius, Göttingen), washed with 10 ml of the same buffer and the radioactivity determined using the LKB-Wallac 81 000 Liquid Scintillation Spectrometer.

3.3. *Affinity labeling experiments*

3.3.1. Subunit specificity of labeling

Analysis of the distribution pattern of radioactivity between subunits isolated from treated 80 S ribo-

Table 1
Influence of uncharged tRNA (*E. coli*) and poly(U) on the binding of $(pU)_8$–RCl to 80 S ribosomes

+ tRNA (0.5 mg/0.2 ml)	+ Poly(U) (0.5 mg/0.2 ml)	$(pU)_8$–RCl bound[a] (pmol)
–	–	66
+	–	117
–	+	46

[a] pmol $(pU)_8$–RCl bound by 130 pmol 80 S ribosomes

Incubation as in fig.1, centrifugation was on a 10–30% sucrose gradient in buffer A in rotor SW 56, Spinco, for 60 min at 50 000 rev./min at 2°C. Ribosomal particles separated from supernatant were precipitated by addition of 0.8 vol. ethanol containing 50 mM $MgCl_2$ and filtered through glass fibre filters GF/A, Whatman, and washed by 4 ml identical buffer/ethanol mixture

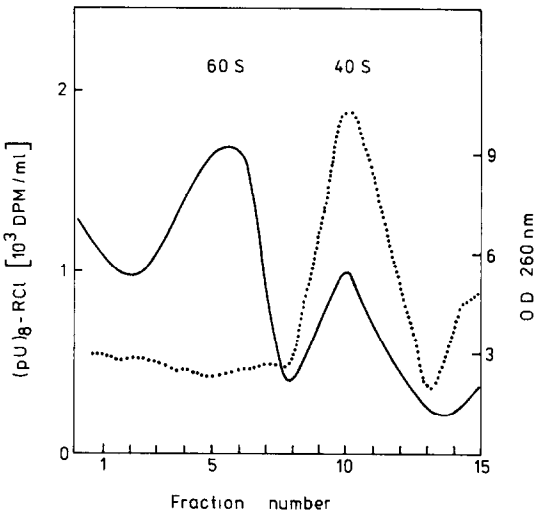

Fig.2. Reaction of $(pU)_n$–RCl with ribosomal subunits. 80 S ribosomes were incubated with $(pU)_n$– RCl and dissociated (section 2): (——) A_{260}; (..) dpm.

somes revealed much higher labeling of the 40 S than of the 60 S subunit (fig.2).

3.3.2. Identification of labeled proteins

Analysis of the radioactivity in the stained spots after two-dimensional electrophoresis of 40 S subunit proteins (fig.3) demonstrates that the highest labeling is associated with ribosomal protein(s) S3/3a. It is not yet known whether one or both of these proteins are

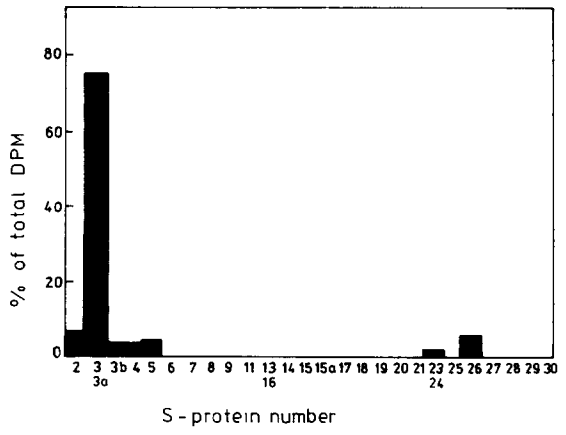

Fig.3. Two-dimensional polyacrylamide gel electrophoresis of small subunit proteins after reaction of $(pU)_n$–RCl with 80 S ribosomes as well as with 40 S subunits. The stained spots were cut out, digested with hyamine and the radioactivity determined as in fig.1.

accessible to the reactive group of the affinity label. On the one hand, the relatively low specific radioactivity of the reagent used necessitates the application of ~2 mg ribosomal protein/gel slab which causes fairly large spots on the gel. On the other hand, due to the extended incubation (20 h) used for the alkylation reaction a partial oxidation of certain ribosomal proteins could not be avoided. In the case of proteins S3 and S3a such reactions produced, at least in some cases, slightly faster moving satellite spots. However, all the radioactivity associated with the area of S3/3a most likely results from substitution of these two proteins. Definite identification of one or both of these proteins as acceptors of the affinity label may result from experiments with specific antibodies that are in preparation.

When 40 S ribosomal subunits were incubated with $(pU)_8$–RCl under the same conditions as complete ribosomes, the bulk of radioactivity was also found in ribosomal protein(s) S3/3a. In this case, however, the size of the satellite spots of S3 and S3a was even somewhat larger than that observed after treatment of 80 S ribosomes.

3.4. *Competition experiments*

When poly(U) as a competitive inhibitor was added to the first incubation mixture prior to $(pU)_8$–RCl, only a very small amount of radioactivity was found in the small ribosomal subunit and the proteins isolated from the small subunit were completely free of radioactive labeling (not shown).

4. Discussion

After UV irradiation of complexes between radioactive poly(U) and 40 S subunits, ribosomal protein S6 was found to be preferentially crosslinked with uridine residues [6]. This result is in accordance with other studies supporting a role of protein S6 in mRNA binding to the ribosome [15,16]. The result presented here is not necessarily contradictory to that above. Whereas in the case of poly(U) any pU can react after UV irradiation with its environment, the reactive group of the affinity label used here is associated with the ribose moiety of the 3′-end of octauridylate and therefore is capable of reacting with the environment at a certain distance from the 2′,3′-position of this terminal ribose. Thus, protein(s) S3/3a are candidates for the contact region of the ribose moieties of mRNA.

Lack of reactivity of $(pU)_8$–RCl with protein S6, however, may also be due to the lack of nucleophilic groups of this protein within the reach of this label or due to formation of an unstable bond at pH 1, for example with carboxylic groups.

According to results obtained [17] by immune electron microscopy of eIF-2/initiation complexes, both proteins, S3 and S6, are located close to each other. Protein S3 furthermore was shown to be located very close to the 3′-end of 18 S RNA [18] and was found to take part in the binding of the complex eIF-2, GTP and Met-$tRNA_f$ during initiation [19] as obvious from crosslinking experiments.

Summarizing the results of experiments on affinity labeling of rat liver ribosomes by $(pU)_8$–RCl as an analogue of mRNA, it can be concluded that protein(s) S3/3a are located, at least with part of the polypeptide chain(s) in close proximity to the mRNA binding region.

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References

[1] Fiser, J., Scheit, K. H., Stoeffler, G. and Kuechler, E. (1975) FEBS Lett. 56, 226–229.
[2] Pongs, O., Stoeffler, G. and Lanka, E. (1975) J. Mol. Biol. 99, 301–315.
[3] Luehrmann, R. and Gassen, H. G. (1976) Eur. J. Biochem. 66, 1–9.
[4] Pongs, O., Petersen, H. U., Grunberg-Manago, M., Lanka, E. and Bald, R. (1979) J. Mol. Biol. 134, 329–345.
[5] Budker, B. G., Grineva, N. I., Karpova, G. G., Kobets, N. D. and Maev, S. P. (1978) Molekul. Biol. 12, 539–545.
[6] Terao, K. and Ogata, K. (1979) J. Biochem. (Tokyo) 86, 605–617.
[7] Mc Conkey, E. H., Bielka, H., Gordon, J., Lastick, S. M., Lin, A., Ogata, K., Reboud, J.-P., Traugh, R. R., Warner, J. R., Welfle, H. and Wool, I. G. (1979) Mol. Gen. Genet. 169, 1–6.
[8] Stahl, J., Böhm, H., Pozdnjakow, V. A. and Girshovich, A. S. (1979) FEBS Lett. 102, 226–229.
[9] Henkel, B., Welfle, H. and Bielka, H. (1974) Acta Biol. Med. Germ. 33, 691–698.
[10] Budker, B. G., Gimautdinova, O. I., Karpova, G. G., Kobets, N. D. and Teplova, N. M. (1976) Molekul. Biol. 10, 340–345.
[11] Welfle, H., Stahl, J. and Bielka, H. (1972) FEBS Lett. 26, 228–232.
[12] Welfle, H., Goerl, M. and Bielka, H. (1978) Mol. Gen. Genet. 163, 101–113.
[13] Stahl, J., Heumann, W., Littmann, G. and Sauerbrey, E. (1966) Acta Biol. Med. Germ. 14, 108–113.
[14] Jones, O. W., Townsend, E. E., Sober, H. A. and Heppel, L. A. (1964) Biochemistry 3, 238–246.
[15] Leader, D. P. (1979) in: Protein Phosphorylation in Regulation (Cohen, P. C. ed) Elsevier/North-Holland, Amsterdam, New York.
[16] Anderson, W. F., Bosch, L., Cohn, W. E., Lodish, H., Merrick, W. C., Weissbach, H., Wittmann, H. G. and Wool, I. G. (1977) FEBS Lett. 76, 1–10.
[17] Bommer, U.-A., Noll, F., Lutsch, G. and Bielka, H. (1980) FEBS Lett. 111, 171–174.
[18] Svoboda, A. J. and Mc Conkey, E. H. (1978) Biochem. Biophys. Res. Commun. 81, 1145–1152.
[19] Westermann, P., Heumann, W., Bommer, U.-A., Bielka, H., Nygard, O. and Hultin, T. (1979) FEBS Lett. 97, 101–104.